

Revised Description of the Fine Structure of *in situ* “Zooxanthellae” Genus *Symbiodinium*

TIMOTHY S. WAKEFIELD^{1,*}, MARK A. FARMER², AND STEPHEN C. KEMPF¹

¹Department of Biological Sciences, Auburn University, Alabama 36849-5414; and ²Center for Advanced Ultrastructural Research, University of Georgia, Athens, Georgia 30602

Abstract. The fine structure of the symbiotic dinoflagellate genus *Symbiodinium* has been well described. All of the published descriptions are based on tissue that was fixed in standard aldehyde and osmium fixatives and dehydrated in an ethanol series before embedding. When the technique of freeze-substitution was used to fix tissue from *Cassiopeia xamachana*, *Aiptasia pallida*, and *Phyllactis flosculifera* and prepare it for embedding, thecal vesicles were revealed within the *in situ* symbionts of all three species. Although these structures have been identified in cultured symbionts, they have never been described in the *in situ* symbionts. A review of the literature has revealed several instances where thecal vesicles were either overlooked or identified incorrectly. Thus the formal description of the genus *Symbiodinium*, which describes the *in situ* symbionts, contains information that is based on artifact and should be revised. A revision of the genus is suggested, and the true nature of these structures and their significance in the symbiotic association are discussed.

Introduction

The symbiotic algae found in the tissues of numerous marine invertebrates and often called “zooxanthellae” have included members of the classes Bacillariophyceae, Cryptophyceae, Dinophyceae, and Rhodophyceae (Rowan, 1998). In recent years, common usage has relegated this term almost exclusively to the description of dinoflagellate symbionts. Loeblich and Sherley (1979) suggested that many of the taxonomically described dinoflagellate symbionts should be assigned to a new genus they termed *Zooxanthella* in recognition of very early work on dinoflagellate symbiosis by Brandt (1881). However, continued studies in

symbiotic dinoflagellate taxonomy have shown that there are, in fact, many species from several genera including *Amphidinium* (Taylor, 1971a; Trench and Winsor, 1987), *Aureodinium* (Anderson and Be, 1976), *Gymnodinium* (Spero, 1987), *Gyrodinium* (Spindler and Hemleben, 1980), *Prorocentrum* (Yamasu, 1988), *Pyrocystis* (Alldredge and Jones, 1973), *Scrippsiella* and *Gloeodinium* (Banaszak *et al.*, 1993). The most frequently occurring of these is the gymnodinioid-like dinoflagellate assigned to the genus *Symbiodinium*, the alga commonly found in a variety of marine invertebrates such as tridacnid clams, certain nudibranch molluscs, reef-building corals, other anthozoans, and scyphozoans.

The first formal description of the dinoflagellate genus *Symbiodinium* was published by Hugo D. Freudenthal (1962). This work provided a detailed histological description of the species *S. microadriaticum* that had been extracted and cultured from a scyphozoan host described as *Cassiopeia* sp. In 1968 Taylor published the preliminary ultrastructural description of dinoflagellate “zooxanthellae” from *Anemonia sulcata*. The author stated that the symbionts were “akin” to *S. microadriaticum*, but that the status of any relationship would remain unknown until an ultrastructural study was performed on the symbionts of *Cassiopeia*. Kevin *et al.* (1969) provided this information in their ultrastructural examination of *S. microadriaticum* from the hosts *Cassiopeia* sp. (Scyphozoa) and *Condylactis* sp. (Anthozoa). They stated that the symbionts from these two genera were the same species as those seen by Taylor, and concluded by amending Freudenthal’s formal description to include the ultrastructural data. Taylor agreed with their conclusions and later identified the dinoflagellates from several species of Tridacnidae as *S. microadriaticum* (Taylor, 1969). In 1987, Trench and Blank published the most recently amended formal description of the genus *Symbio-*

dinium, and described three new species in the genus. For the most part, all four of these papers describe similar ultrastructural features. This is not surprising considering that both Kevin and Trench describe *S. microadriaticum* from its definitive host *Cassiopeia*, and that all four used a standard chemical fixation that included buffered glutaraldehyde and osmium followed by a dehydration series in preparing their tissues for the transmission electron microscope.

In the following paper, we further amend the ultrastructural description of the genus *Symbiodinium* on the basis of the use of freeze-substitution, an alternative fixation technique that provides better preservation of fine structure. The symbionts from three hosts were examined: two anthozoans—*Aiptasia pallida* (*S. bermudense*) and *Phyllactis flosculifera* (*Symbiodinium* sp.)—and the definitive scyphozoan host *Cassiopeia xamachana* (*S. microadriaticum*). This has resulted in a more clearly resolved ultrastructure revealing previously undescribed features in the *in situ* symbionts. These results suggest an alternative hypothesis for the origin of the multilayered “periplast” or “symbiosome membrane” that is prevalent around the dinoflagellate symbionts found in invertebrate hosts.

Materials and Methods

Animal collection and care

Specimens of *Cassiopeia xamachana*, *Aiptasia pallida*, and a second anthozoan species identified as *Phyllactis flosculifera* (Fautin, University of Kansas, pers. comm.) were collected in the Florida Keys and transported to Auburn University, Alabama. These species were maintained in established 150-gallon aquaria filtered with both under-gravel and external trickle filtration. Artificial seawater was prepared with Reef Crystals (Aquarium Systems) and deionized water at about 30 ppt. The aquaria were maintained at 25°–30° C on a 12/12 h light/dark cycle, and the animals were fed with newly hatched brine shrimp nauplii every other day.

Fixation and embedding

Anthozoan procedure. Individual specimens of *A. pallida* and *P. flosculifera* were transferred to small glass bowls containing 0.45- μ m Millipore-filtered artificial seawater (MFAS) and were allowed to acclimate, attach to the glass substratum, and fully expand. Small pieces of tentacle (<10 mm in length, but very thin) were excised from individuals of both species and suspended from small lengths of platinum wire. The tissues were then plunge-frozen in liquid propane (Menco, 1986) and transferred to liquid nitrogen. The frozen tissues were transferred to glass vials containing a 2% osmium tetroxide-acetone solution that had been previously cooled to -80° C. The vials were held within a

precooled, methanol-saturated sponge, and the tissue samples were left at -80° C for 48–72 h before being transferred to -20° C. Specimens remained at -20° C for 12–24 h and were then moved to 4° C for an additional 12–24 h before being returned to room temperature. While the specimens were being transferred from -80° C to room temperature, a gradual warming was achieved by moving the entire methanol-saturated sponge containing the vials from one environment to another. After reaching room temperature, the tissue samples were removed, and the platinum wire was discarded. Tissues were washed in 100% acetone and were either transferred to 100% propylene oxide before infiltration, or were immediately infiltrated and embedded in Poly-Bed 812 plastic.

Scyphozoan procedure. Several attempts were made to fix *Cassiopeia xamachana* symbionts *in situ* using the procedure described above, but all resulted in very poor fixation. We speculate that the copious amounts of mucus produced by the scyphozoan tissue acted as an insulator against the rapid freezing of the tissue. Thus the following, more successful, procedure was used. Six large specimens of *C. xamachana* were transferred to a large bowl of MFAS. The oral arms of each specimen were excised and discarded. With a glass slide, the external symbiotic layer of tissue from the oral side of the specimen's bell was scraped free from the underlying mesoglea and collected. The remaining mesoglea and aboral ectoderm was discarded. The collected symbiotic tissues were homogenized in MFAS using a Virtis Handishear tissue homogenizer. The tissue homogenate was centrifuged at $\sim 250 \times g$ for 3 min to pellet the separated symbiosomes, and the supernatant was discarded. Small ($\sim 1 \text{ mm}^2$) samples of the pelleted symbiosomes were placed on thin strips of filter paper and then immediately plunge-frozen, fixed, and embedded in the manner described above for the anthozoan tissue.

Standard fixation and embedding. As a control for the ultra-rapid freezing and freeze-substitution fixation process, a standard aldehyde-osmium fixation at room temperature was performed on each of the three symbiotic cnidarian tissues. Briefly, small pieces (<10 mm in length) of tentacle from symbiotic *A. pallida* and *P. flosculifera*, as well as small portions (<10 mm²) of the bell margin from *C. xamachana*, were removed from living specimens and immersed in 2.5% glutaraldehyde in 20 mM Millonigs phosphate buffered saline (MPBS), pH 7.6. The tissue was allowed to fix for 1 h, rinsed twice for 5 min in 20 mM MPBS, and then postfixed in 2% OsO₄ in 1.25% NaHCO₃ for 1 h. Residual OsO₄ was rinsed away in two 5-min rinses of 1.25% NaHCO₃. Tissues were then dehydrated in an ethanol series (30%, 50%, 70%, 85%, 90%, 95%, $3 \times 100\%$), followed by three rinses in propylene oxide (10 min in each solution), all at room temperature. Tissues were then infiltrated and embedded in Poly-Bed 812 plastic in the same manner as the freeze-substituted tissues.

Sectioning and staining

Ultrathin sections of each kind of tissue were cut with a Reichert-Jung Ultracut E ultramicrotome and collected on Formvar-coated slot grids. Ultrathin sections were stained with lead citrate and uranyl acetate and were examined on a Zeiss EM-10 transmission electron microscope (TEM).

Measurements

Cell wall layers and thecal vesicles were measured directly from TEM negatives with a hand lens, light box, and metric ruler. A total of 100 thecal vesicles were measured for both *Symbiodinium bermudense* (*Aiptasia pallida*) and *Symbiodinium* sp. (*Phyllactis flosculifera*). In those symbionts that contained more than 10 vesicles, only 10 were measured. Other symbionts did not contain 10 clearly defined vesicles; thus a total of 15 *S. bermudense* and 13 *Symbiodinium* sp. were used to obtain the 100 total vesicles measured.

Even using the alternative method, thecal vesicle preservation for *S. microadriaticum* from *Cassiopeia xamachana* was still inferior to that of the symbionts from the other hosts. In this species, only those thecal vesicles that could be clearly identified were measured, resulting in a lower "n" value from 26 cells for the vesicles measured in *S. microadriaticum*. Cell wall layers (*S. bermudense*) and total cell wall thickness (all symbiont species) were measured as described above. Conversions from millimeters to micrometers or nanometers were made using a published nomogram (Ghadially *et al.*, 1981).

Results

Many aspects of the ultrastructure of freeze-substituted *in situ* symbionts show good correspondence with previously published descriptions of the genus *Symbiodinium* (Taylor, 1968, 1969; Kevin *et al.*, 1969; Trench and Blank, 1987). The multi-lobed chloroplast is peripherally located, with parallel rows of thylakoids usually arranged in groups of three. A single, stalked pyrenoid may be seen emerging from the chloroplast. Its membrane appears to be continuous with the chloroplast envelope with no invading thylakoids, and a relatively thick starch coat surrounds the entire structure. A single large, irregularly shaped, granular accumulation body is present in many cells. Typical dictyosomes and mitochondria also occur, as do many lipid vacuoles and some calcium oxalate crystals. Large, well-preserved "fibrous bodies" are present in many of the symbionts (Dodge, 1967). The nucleus of the symbionts is distinct and recognizable, but the compact spirals of chromatin typically seen in the mesokaryotic nucleus are not always visible.

The freeze-substitution process did, however, reveal some previously undescribed features of the *in situ* symbionts, as well as differences among *Symbiodinium* species.

The "amorphous layer" of the periplast described by Kevin *et al.* (1969) has been correctly identified as the continuous, vegetative cell wall of the symbiont (Trench and Blank, 1987). However, our results (Fig. 1) indicate that in *Symbiodinium bermudense* this structure is in fact composed of three distinct layers: an outer layer that appears to be membranous (OL), an electron-dense middle layer (EDL), and a less dense inner layer (IL). As can be seen in Table 1, the greatest variation in layer width was seen in the IL, which ranged from 30 to 300 nm. This variation is evident in nonmitotic cells (Fig. 2), but it is most common in recently divided cells, where the OL and EDL retain similar thickness circumferentially around the divided cells, while the IL

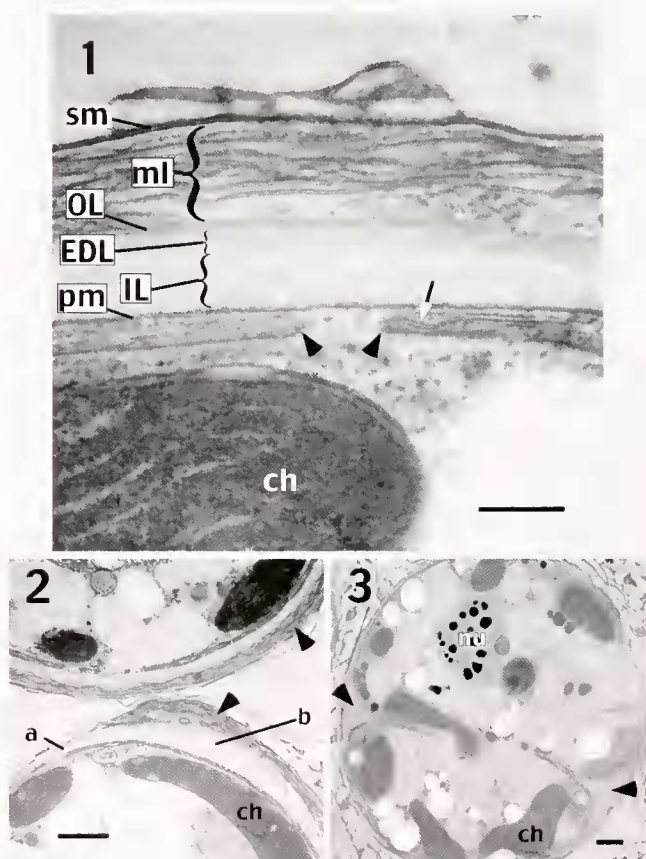


Figure 1. *Symbiodinium bermudense* within the host *Aiptasia pallida*. The cell wall of the symbiont shows three distinct regions beneath the multiple layers of membranous material that surround it. ch = chloroplast; EDL = electron dense cell wall layer; IL = inner cell wall layer; ml = multiple layers of symbiosome membrane; OL = membranous outer layer; pm = algal cell plasma membrane; sm = outer symbiosome membrane; arrow = thecal plate; arrowheads indicate thecal vesicles. Scale bar = 100 nm.

Figure 2. *Symbiodinium bermudense* within host *Aiptasia pallida*. Note the difference in thickness of the cell wall inner layer between points a and b; ch = chloroplast; arrowheads indicate accumulations of symbiosome membrane outside of vegetative cell wall. Scale bar = 500 nm.

Figure 3. Dividing *Symbiodinium bermudense* within *Aiptasia pallida*. ch = chloroplast; nu = nucleus; arrowheads indicate areas of thickening of inner layer of the cell wall along the division furrow. Scale bar = 1 μ m.

Table 1

Cell wall layer thickness, total cell wall thickness, and thecal vesicle thickness (all measurements in nanometers, mean $\pm$ std. dev.)

Symbiont species	IL	EDL	OL	Total thickness (n)	Thecal vesicle thickness (n)
<i>Symbiodinium</i> sp.	—	—	—	137.8 $\pm$ 65.5 (100)	34.3 $\pm$ 7.8 (100)
<i>S. microadriaticum</i>	—	—	—	108.4 $\pm$ 37.7 (100)	38.8 $\pm$ 7.3 (50)
<i>S. bermudense</i>	96.7 $\pm$ 48.4	40.4 $\pm$ 7.7	10.6 $\pm$ 1.4	147.7 $\pm$ 49.9 (100)	34.9 $\pm$ 8.5 (100)

EDL = electron dense layer, IL = inner layer, OL = outer layer; — indicates no visible layers.

becomes increasingly thicker in the region of the division furrow (Fig. 3). *Symbiodinium* sp. from *P. flosculifera* and *S. microadriaticum* lacked this three-layered structure, the entire cell wall being similar in consistency to the IL layer of *S. bermudense*.

A typical multilayered symbiosome membrane could be seen around the symbionts of all three host animals, but was most prevalent around *S. bermudense* (Figs. 1, 2, 4). The membranes that completely surrounded most symbionts seemed to be distributed evenly, but in some cases a disproportionate number of membranes were located to one side of the symbiont (Figs. 2, 4).

Just inside of the cell wall is the continuous cell membrane. Below this cell membrane, the freeze-substitution process has revealed distinct thecal vesicles. These vesicles are most prominent in *Symbiodinium bermudense* (Figs. 4, 5) and the *Symbiodinium* sp. (Figs. 6, 7), but they are also present in *S. microadriaticum* (Figs. 8, 9). In TEM sections, each vesicle is membrane bound and has rounded edges at

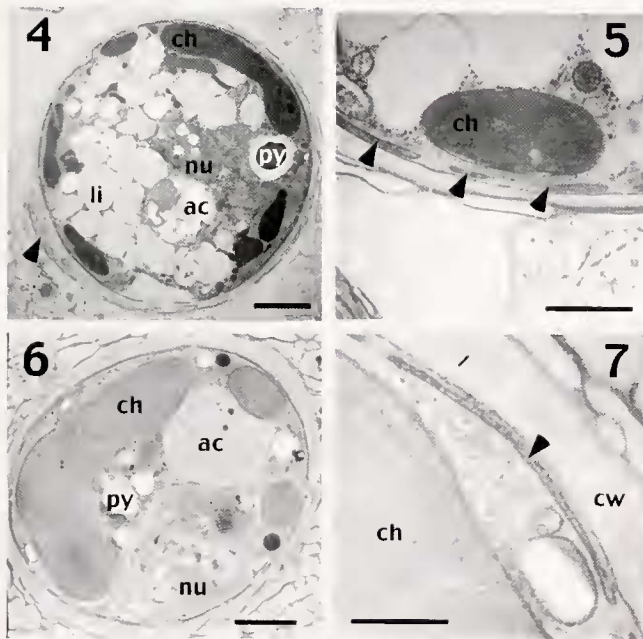


Figure 4. *Symbiodinium bermudense* within *Aiptasia pallida*. ac = accumulation body; ch = chloroplast; li = lipid vacuole; nu = nucleus; py = pyrenoid; arrowhead indicates large number of membranes on only one side of symbiont. Scale bar = 2 μ m.

Figure 5. Thecal vesicles in *Symbiodinium bermudense*. ch = chloroplast; arrowheads indicate individual thecal vesicles. Scale bar = 500 nm.

Figure 6. *Symbiodinium* sp. within the host *Phylactis flosculifera*. ac = accumulation body; ch = chloroplast; nu = nucleus; py = pyrenoid. Scale bar = 1 μ m.

Figure 7. Thecal vesicle in *Symbiodinium* sp. from *Phylactis flosculifera*. ch = chloroplast; cw = cell wall; arrow = algal cell plasma membrane; arrowhead = thecal vesicle. Scale bar = 300 nm.

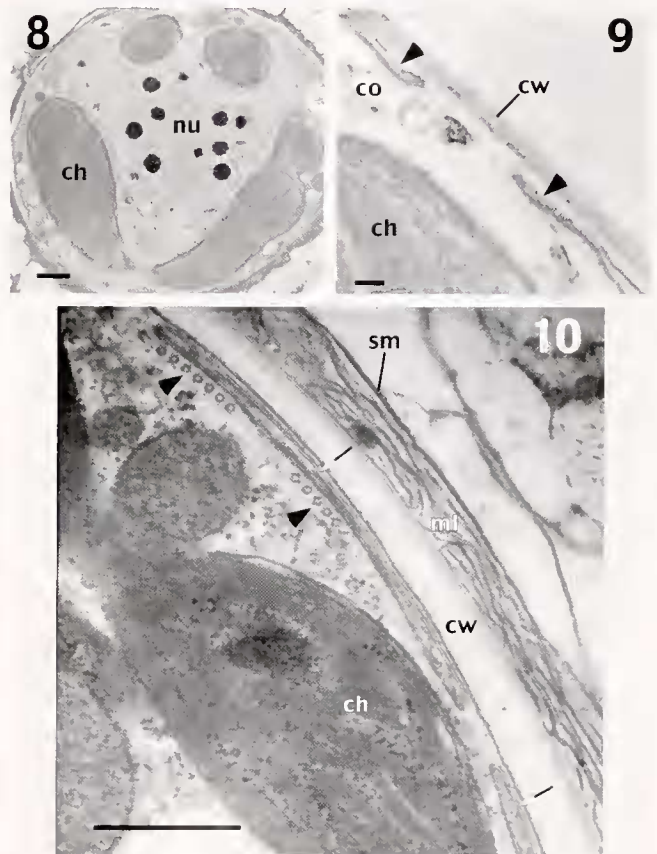


Figure 8. *Symbiodinium microadriaticum* from the host *Cassiopeia xamachana*. ch = chloroplast; nu = nucleus. Scale bar = 500 nm.

Figure 9. Thecal vesicles in *Symbiodinium microadriaticum*. ch = chloroplast; co = calcium oxalate crystal; cw = cell wall; arrowheads indicate thecal vesicles. Scale bar = 100 nm.

Figure 10. *Symbiodinium bermudense* within host *Aiptasia pallida*. ch = chloroplast; cw = cell wall; ml = multiple layers of symbiosome membrane; sm = outer symbiosome membrane; arrows identify thecal vesicles; arrowheads indicate linear array of microtubules beneath thecal vesicles. Scale bar = 300 nm.

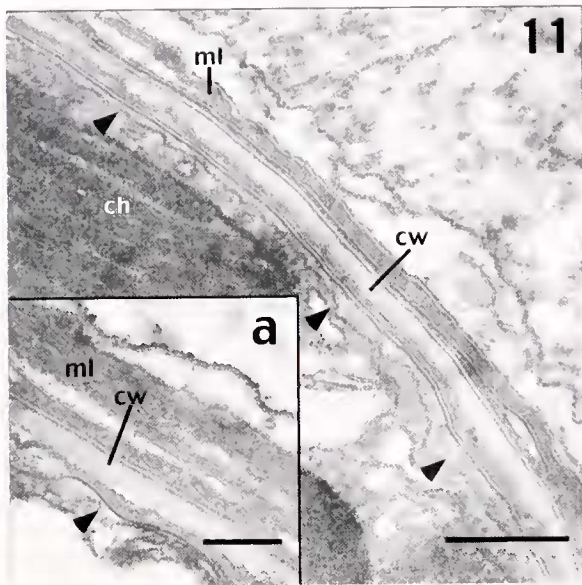


Figure 11. *Symbiodinium bermudense* within host *Aiptasia pallida*. Standard glutaraldehyde-osmium tissue fixation. Note scrolled membrane in region where thecal vesicles should be (arrowheads). ch = chloroplast; cw = cell wall; ml = multiple layers of symbiosome membrane; arrowheads identify area where thecal vesicles should be located. Scale bar = 200 nm. Inset (a) is a higher magnification of an additional symbiont showing layered membranes internal to the cell wall. Scale bar = 100 nm.

each end, with a distinct cytoplasmic separation between adjacent vesicles. Internal thecal plates were apparent within some vesicles of *S. bermudense* (Fig. 1, arrow). The enclosed thecal plate lies separate from the vesicle membrane. The overall thickness of the vesicles for each species is reported in Table 1. Since serial sections of an entire cell were not cut, we could not determine whether the differing widths of the vesicles reflect differences among species, large and small vesicles, or different vesicle profiles. In several micrographs, a linear array of microtubules was present underlying the vesicles (Fig. 10).

Standard aldehyde-osmium fixed tissues of all three species of symbiont failed to show many of the fine structures seen in the freeze-substituted tissue. The multiple-layered symbiosome membrane was apparent; but only the OL and the IL of the cell wall were seen in *S. bermudense*, and all three species failed to show any thecal vesicles or plates. Instead, there appeared to be several layers of membrane beneath the vegetative cell wall (e.g., Fig. 11, 11a).

Discussion

A multiple-layered cell wall in *Symbiodinium bermudense* has been previously described by Palincsar *et al.* (1988; they identified the symbiont of *Aiptasia pallida* as *S. microadriaticum* at that time; however, the species has subsequently been redescribed as *S. bermudense* [see Trench, 1993]). The description by Palincsar *et al.* (1988)

included four layers. From the inside out, the layers were described as a vesicular layer immediately outside the symbiont's plasma membrane; a thick homogeneous finely granular layer; and a "line-thin" dark layer, overlaid by an even thinner membranous layer. They also reported a wide variation in cell wall structure, with some cells displaying an almost entirely homogeneous, finely granular wall and others an almost entirely vesicular wall. The presence of multiple-layered cell walls is not surprising since similar cell wall features have been identified in other dinoflagellate species (Morrill and Loeblich, 1981). Bricheux *et al.* (1992) identified a four-layered pellicle in the free-living dinoflagellate *Glenodinium foliaceum*. The two layers immediately outside of the cell membrane (identified as the homogeneous layer and the dense layer) strongly resemble the IL and the EDL we see in species of *Symbiodinium*. The homogeneous layer in both *G. foliaceum* and *Peridinium balticum*, a symbiont-containing dinoflagellate, has been experimentally shown to contain cellulose (Morrill and Loeblich, 1981; Loeblich, 1984). However, the cellulose in this layer was assumed to be in an amorphous or low crystalline state, since no cellulose fibers have been identified with the electron microscope. On the other hand, the dense layer of *Heterocapsa niei* and other dinoflagellates has been suggested to contain sporopollenin, a very resistant plant terpenoid that has been demonstrated to be insoluble, even in hot ethanolamine (Loeblich, 1970; Morrill and Loeblich, 1981). Although the exact chemical nature of the cell wall of *Symbiodinium* is unknown, Markell *et al.* (1992) reported that the cell walls of *S. microadriaticum*, *S. kawagutii*, and *S. pilosum* did contain cellulose, an observation based on cellulase digestion of isolated cell walls. They also reported that in intact symbionts, either living or glutaraldehyde fixed, cellulase was ineffective in digesting the cell wall. If a part of *Symbiodinium*'s cell wall, such as the EDL, is composed of an extremely insoluble component, such as sporopollenin, the IL may have been unaffected by the cellulase simply because the enzyme could not penetrate to this layer in intact cells.

The current formal description of the genus *Symbiodinium* (Trench and Blank, 1987) states that the cell wall of the coccoid stage varies in thickness. It also states that this variation depends on the life history of the cell. This fact is supported by Bricheux *et al.* (1992), who found that the thickness of the cell wall of the free-living dinoflagellate *G. foliaceum* was relatively thin following ecdysis of the thecal armor; however, as the vegetative stage of the life history progressed, the cell wall became progressively thicker, with the largest amount of change seen in the inner homogeneous layer (IL). Similarly, our study has shown that, although the OL and EDL certainly contribute to the overall thickness of the cell wall, the thickness of the IL has the most variation.

The presence of the thecal vesicles within the symbionts is not in itself surprising, since Loeblich and Sherley (1979)

described the thecate nature of the motile stage of *S. microadriaticum*; but this is the first report that thecal vesicles are present within *in situ* coccoid symbionts. The formal description of the genus *Symbiodinium* describes the coccoid symbionts as having a "continuous cell wall . . . underlain by a series of membranes" (Trench and Blank, 1987). We found no such membranes in our ultrastructural investigations of freeze-substituted symbionts, but instead found distinct thecal vesicles in this region. We did see apparent membranes like those described by Trench and Blank (1987) when the tissues were fixed with standard aldehyde-osmium fixations. Since thecal vesicles are well described in other dinoflagellate species (Dodge and Crawford, 1970; Bricheux *et al.*, 1992; Hohfeld and Melkonian, 1992), have been reported in *in vitro* cultured *Symbiodinium* (Taylor, 1971b; Loeblich and Sherley, 1979; Trench and Blank, 1987), and are not artifactual in nature, we conclude that the multiple membranes reported in the formal *in situ* description of the genus *Symbiodinium* (Trench and Blank, 1987) were the result of a fixation artifact.

Although this is the first report identifying the presence of thecal vesicles within the *in situ* coccoid symbionts, review of the ultrastructural literature relating to the genus *Symbiodinium* reveals apparent thecal vesicles in the figures from various published papers. In Taylor's (1968) original paper on symbiont ultrastructure, figure 3 clearly shows what appear to be thecal vesicles beneath the cell wall of the coccoid stage. However, Taylor states that the vesicles are formed in older symbionts as two of the multiple membrane layers undergo a series of infoldings to form these isolated and discrete vesicles, and he does not associate them with the thecal covering of the motile stage. Kevin *et al.* (1969) show, in their figure 5, an *in situ* symbiont that clearly has thecal vesicles beneath the plasma membrane; however, these structures are neither identified nor mentioned in the text. In 1970, Dodge and Crawford reported that two membranes, "the outer of which appear to be folded over in places," surrounded the cytoplasm of the symbionts from *Anemonia sulcata*. Their plate 7 also shows distinct thecal vesicles inside the cell wall (Dodge and Crawford, 1970). A paper by Tripodi and Santisi (1982) describes the ultrastructure of *S. microadriaticum* within the octocoral *Eunicella stricta*. In their figures 4, 5, and 6, the electron micrographs show distinct thecal vesicles beneath and adjacent to the cell membrane of the *in situ* symbionts. Their figure 8, a line drawing describing the cell covering of the symbionts, also clearly shows distinct thecal vesicles. However, the figure captions, as well as the text, describe these vesicles as profiles of the endoplasmic reticulum and once again do not associate them with the thecate motile stage.

In all of these reports, the symbiotic tissues underwent standard fixation in 3%–4% glutaraldehyde followed by post-fixation in 1%–2% osmium tetroxide. Therefore, the thecal vesicles can be visualized within the *in situ* sym-

bionts using this type of fixation; but the results in these papers are the exception rather than the rule. It would appear that, during a standard chemical fixation, if conditions are not right (osmolality, temperature, length of fixation, age of fixative, *etc.*), the fragile thecal vesicles rupture and combine with the plasma membrane to take on the appearance of multiple or scrolled layers of membrane beneath the cell wall. Our results suggest that a more reliable way of visualizing this fine structure is by use of the freeze-substitution process.

The presence of thecal vesicles beneath the plasma membrane of the *in situ* symbionts lends further credence to an interesting hypothesis. In 1971, Taylor reported the development of flagellar structures in the *in situ* symbionts of the cnidarian *Velella velella* (pl. IIIA) and the flatworm *Amphiscolops langerhansii* (pl. IIIB). In 1980, Schoenberg and Trench also reported the presence of flagellar structures in *in situ* *Symbiodinium microadriaticum* from *Protopalycha* sp. (figure 8, plate 5). They state that the sporadic appearance of these flagellar structures could represent the transient production of motile zoospores *in situ*.

The production of motile gymnodinioid zoospores is a daily occurrence in log-phase cultures of *Symbiodinium in vitro*. Within the cell wall of the vegetative mother cell, a metamorphosis occurs that results in the development of a characteristically gymnodinioid motile cell with thecae, hypocone, epicone, and longitudinal and transverse flagellae in their respective grooves or furrows (Freudenthal, 1962; Loeblich and Sherley, 1979). Motile stages often develop after the cell has divided into daughter cells, but they can also develop without any previous cell division (Schoenberg and Trench, 1980). When mature, the zoospores escape from the mother cell by some unknown mechanism to swim freely within the culture medium (Trench and Blank, 1987). After a relatively short motile stage the cells spontaneously undergo ecdysis, a process by which they shed their thecae and flagella, again by some unknown mechanism, and settle to the substratum, where they enter the longer vegetative phase of their life cycle.

The mitotic rate of *in situ* symbionts is decreased in comparison to cultured symbionts. Fitt and Trench (1983) determined that, within cultures of *Symbiodinium microadriaticum*, as many as 25% of the cells could be dividing, but Palincsar *et al.* (1988) determined the mitotic rate of *S. bermudense* within *A. pallida* to be as low as 1.2%. Although the mitotic rate *in situ* is decreased, it is not halted. We hypothesize that a similar sort of delay occurs in the ecdysis cycle and that, on a slower and perhaps irregular schedule, ecdysis continues *in situ*. This would explain the discrepancies between our cell wall description and that of Palincsar *et al.* (1988). The variations in cell wall structure that they describe (totally

granular, to half granular/half vesicular, to almost completely vesicular) could be explained by symbionts caught in varying stages of the ecdysis. Similarly, such a cycle would also result in the sporadic appearance of flagella, as reported in some *in situ* symbionts (Taylor, 1971a, b; Schoenberg and Trench, 1980), and the presence of thecal vesicles, as reported in this paper.

We also suggest that the delayed ecdysis cycle could be responsible for the multiple layers of apparent membranes that are often found just outside of the vegetative cell wall of *in situ* symbionts. Unlike those of cultured symbionts, the shed thecal plates and outer plasma membrane of *in situ* symbionts would not be discarded but would accumulate inside of the symbiosome membrane (Fig. 12). Such shed plates would appear as the accumulations of the apparent membranous material (ml) that has been observed between the outer symbiosome membrane and the vegetative cell wall of the symbionts (Taylor, 1968; Kevin *et al.*, 1969; Schoenberg and Trench, 1980; Tripodi and Santisi, 1982;

Colley and Trench, 1983; Blank, 1987; Trench and Blank, 1987; Trench and Winsor, 1987; Palincsar *et al.*, 1988; Rands *et al.*, 1993).

These accumulations of apparently membranous material were first described by Taylor (1968), who ascribed their origin to both the algal cell and the host, but gave no specific structures from which they might arise. Later, Kevin *et al.* (1969) redefined the location of the membranes surrounding the algal cell, but once again failed to clearly state their origin. This uncertainty as to the origin of these membranes has continued throughout the literature, with some authors attributing it to the host (Tripodi and Santisi, 1982; Colley and Trench, 1983; Palincsar *et al.*, 1988; Rands *et al.*, 1993), and others to the algal cell (Schoenberg and Trench, 1980; Trench and Blank, 1987). Specifically, Trench and Blank (1987) reported that the outer layer of the cell wall is periodically "sloughed off" the surface and often produces a "scroll-like" appearance in sections. However, they do not offer any evidence to support this process as the origin of the multiple membranous layers, nor do they offer any suggestions about how this sloughed layer might be regenerated outside of the continuous cell wall. Our hypothesis of a continuing *in situ* ecdysis cycle as the origin of the apparent membranes (see Fig. 12) is based on the presence of symbiont thecal vesicles *in situ*, and on a known event within the life cycle of the symbiont. As such, it does not require the proposal of another "unknown mechanism" to explain how additional membranes would be added to those already present around the symbiont.

There is another question that must be addressed if our hypothesis is correct. If a delayed ecdysis cycle is continuing within the host cell symbiosome, then in addition to the shedding of the theca and plasma membrane, there must also be a shedding of the cell wall. If the thecal vesicles are retained within the host membrane, what happens to the cell wall material? Although this is a valid question, it is not unique to our hypothesis. The same question can be asked of mitotically active, vegetative cells *in situ*.

It has been assumed that, at the conclusion of a mitotic event, the symbiont daughter cells within the same symbiosome membrane are separated by invading extensions of symbiosome membrane and host cytoplasm (Reisser, 1992). Following division, each new daughter cell produces a new cell wall within the old cell wall of the parent cell (Taylor, 1968; Kevin *et al.*, 1969). Thus, if the "old" cell wall were not degraded in some way, each host symbiosome would contain remnants of cell wall material from previous mitotic events. Because such remnants have not been observed, the old cell wall material must be degraded, perhaps by enzymes released from the symbiont itself. As was mentioned previously, the release of motile zoospores from within the parent cell wall is controlled by an unknown mechanism, presumably enzy-

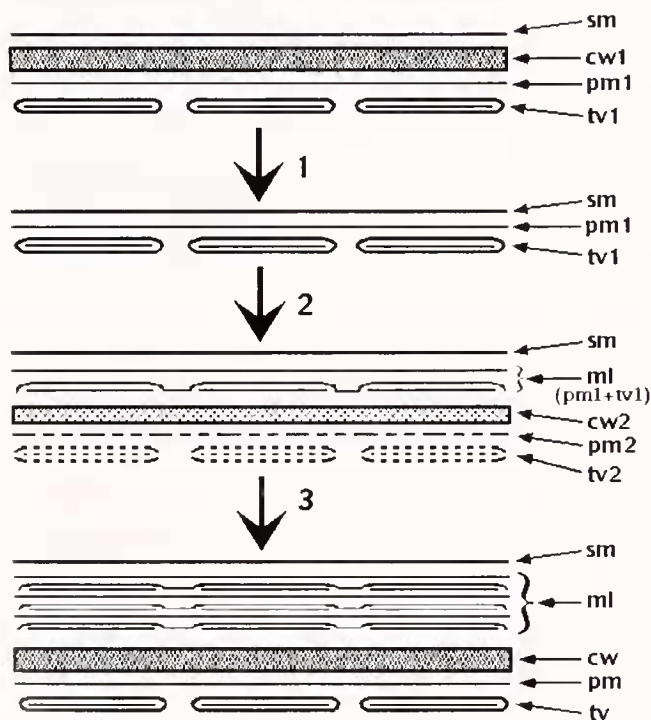


Figure 12. A hypothesis for how multiple layers of membrane could accumulate within symbiosome membrane: step 1. Vegetative cell gives rise to motile "thecate" cell; cell wall disappears. step 2. Motile cell undergoes ecdysis and sheds plasma membrane and thecal vesicles as the cell wall is reformed and new thecal vesicles form beneath it. step 3. After several cycles, accumulated material from algal plasma membrane and thecal vesicles appear as multiple membranes between the outer symbiosome membrane and the vegetative algal cell wall. cw = cell wall; ml = multiple layers of membrane; pm = symbiont plasma membrane; sm = surface of symbiosome membrane; tv = thecal vesicles. (Figure adapted from Hohfeld and Melkonian, 1992.)

matic in nature. Perhaps this same mechanism is responsible for the degradation of the cell wall within the host symbiosome during the mitotic event, with the outer symbiosome membrane retaining active enzymes in the vicinity of the discarded cell wall. In the case of a retarded ecdysis cycle, if the thecal plates differ in composition from the cell wall and thus are not subject to similar enzymatic breakdown, they could accumulate as the multiple layers of membranous material found between the symbiosome membrane and symbiont cell wall *in situ*.

These membranes contribute to the membranous structure of the symbiosome and are part of the boundary between host and symbiont. All "communication" between host and symbiont, transport of gasses, and translocation of photosynthetically fixed carbon must occur through, and in conjunction with, these membranes (Rands *et al.*, 1993). Thus their origin and their role in these events is of great importance.

Acknowledgments

We thank Dr. Daphne Fautin for her aid in identifying the anthozoan species *Phyllactis flosculifera*. We also thank Dr. William Fitt and the Key Largo Marine Research Laboratory, Dr. Mike Miller and the Auburn University Biological Electron Microscope Imaging Facility, and the University of Georgia's Center for Advanced Ultrastructural Research for their assistance in this research. This work was supported by NSF #9018698 (SCK), NSF-BIR #9220230 (SCK), ONR #3231114 (SCK), and a grant from the Alabama Agricultural Experiment Station (SCK) Journal # 6-996072.

Literature Cited

- Allredge, A. L., and B. M. Jones. 1973. *Hastigerina pelagica*: foraminiferal habitat for planktonic dinoflagellates. *Mar. Biol.* **22**: 131–135.
- Anderson, O. R., and A. W. H. Be. 1976. The ultrastructure of a planktonic foraminifer, *Globigerinoides sacculifer* (Brady), and its symbiotic dinoflagellates. *J. Foram. Res.* **6**: 1–9.
- Banaszak, A. T., R. Iglesias-Prieto, and R. K. Trench. 1993. *Scrippsiella velellae* sp. nov. (Peridinales) and *Gloeodinium viscum* sp. nov. (Phytodiniales), dinoflagellate symbionts of two hydrozoans (Cnidaria). *J. Phycol.* **29**: 517–528.
- Blank, R. J. 1987. Cell architecture of the dinoflagellate *Symbiodinium* sp. inhabiting the Hawaiian stony coral *Montipora verrucosa*. *Mar. Biol.* **94**: 143–155.
- Brandt, K. 1881. Über das Zusammenleben von Thieren und Algen. *Verh. Physiologischen Ges. Berlin*. 1881–1882: 22–26.
- Bricheux, G., D. G. Mahoney, and S. P. Gibbs. 1992. Development of the pellicle and thecal plates following ecdysis in the dinoflagellate *Glenodinium foliaceum*. *Protoplasma* **168**: 159–171.
- Colley, N. J., and R. K. Trench. 1983. Selectivity in phagocytosis and persistence of symbiotic algae by the scyphistoma stage of the jellyfish *Cassiopeia xamachana*. *Proc. R. Soc. Lond. B.* **219**: 61–82.
- Dodge, J. D. 1967. Fine structure of the dinoflagellate *Aureodinium pigmentosum* gen. et sp. nov. *Br. Phycol. Bull.* **3**: 327–336.
- Dodge, J. D., and R. M. Crawford. 1970. A survey of thecal fine structure in the Dinophyceae. *Bot. J. Linn. Soc.* **63**: 53–67.
- Fitt, W. K., and R. K. Trench. 1983. The relation of diel patterns of cell division to diel patterns of motility in the symbiotic dinoflagellate *Symbiodinium microadriaticum* Freudenthal in culture. *New Phytol.* **94**: 421–432.
- Freudenthal, H. D. 1962. *Symbiodinium* gen. nov. and *Symbiodinium microadriaticum* sp. nov., a zooxanthella: taxonomy, life cycle, and morphology. *J. Protozool.* **9**: 45–52.
- Ghadially, F. N., P. Jackson, and J. Innor. 1981. A nomogram for electron microscopists. *J. Submicrosc. Cytol.* **13**: 95–96.
- Hohfeld, I., and M. Melkonian. 1992. Amphiesmal ultrastructure of dinoflagellates: a reevaluation of pellicle formation. *J. Phycol.* **28**: 82–89.
- Kevin, M. J., W. T. Hall, J. J. A. McLaughlin, and P. A. Zahl. 1969. *Symbiodinium microadriaticum* Freudenthal, a revised taxonomic description, ultrastructure. *J. Phycol.* **5**: 341–350.
- Loeblich, A. R. I. 1970. The amphiesma or dinoflagellate cell covering. Pp. 867–929 in *Proceedings of the North American Paleontological Convention*, E. L. Yochelson, ed. Allen Press, Chicago.
- Loeblich, A. R. I. 1984. Dinoflagellate physiology and biochemistry. Pp. 299–342 in *Dinoflagellates*, D. L. Spector, ed. Academic Press, New York.
- Loeblich, A. R. I., and J. L. Sherley. 1979. Observations on the theca of the motile phase of free-living and symbiotic isolates of *Zooxanthella microadriatica* (Freudenthal) comb. nov. *J. Mar. Biol. Assoc. UK* **59**: 195–205.
- Markell, D. A., R. K. Trench, and R. Iglesias-Prieto. 1992. Macromolecules associated with the cell walls of symbiotic dinoflagellates. *Symbiosis* **12**: 19–31.
- Menco, B. P. M. 1986. A survey of ultra-rapid cryofixation methods with particular emphasis on applications to freeze-fracturing, freeze-etching, and freeze-substitution. *J. Electron Microsc. Tech.* **4**: 177–240.
- Morrill, L. C., and A. R. I. Loeblich. 1981. The dinoflagellate pellicular wall layer and its occurrence in the division Pyrrhophyta. *J. Phycol.* **17**: 315–323.
- Palincsar, J. S., W. R. Jones, and E. E. Palincsar. 1988. Effects of isolation of the endosymbiont *Symbiodinium microadriaticum* (Dinophyceae) from its host *Aiptasia pallida* (Anthozoa) on cell wall ultrastructure and mitotic rates. *Trans. Am. Microsc. Soc.* **107**: 53–66.
- Rands, M. L., B. C. Loughman, and A. E. Douglas. 1993. The symbiotic interface in an alga-invertebrate symbiosis. *Proc. R. Soc. Lond. B.* **253**: 161–165.
- Reisser, W. 1992. Basic mechanisms of signal exchange, recognition, specificity, and regulation in endosymbiotic systems. Pp. 657–674 in *Algae and Symbioses*, W. Reisser, ed. Biopress, Bristol, England.
- Rowan, R. 1998. Diversity and ecology of zooxanthellae on coral reefs. *J. Phycol.* **34**: 407–417.
- Schoenberg, D. A., and R. K. Trench. 1980. Genetic variation in *Symbiodinium* (= *Gymnodinium*) *microadriaticum* Freudenthal, and specificity in its symbiosis with marine invertebrates. II Morphological variation in *Symbiodinium microadriaticum*. *Proc. R. Soc. Lond. B.* **207**: 429–444.
- Spero, H. J. 1987. Symbiosis in the planktonic foraminifera, *Obolina universa*, and the isolation of its symbiotic dinoflagellate *Gymnodinium beii* sp. nov. *J. Phycol.* **23**: 307–317.
- Spindler, M., and C. Hemleben. 1980. Symbionts in planktonic foraminifera (Protozoa). Pp. 133–140 in *Endocytobiology, Endosymbiosis, and Cell Biology*, W. Schwemmler and H. E. A. Schenk, eds. Walter de Gruyter, Berlin.

- Taylor, D. L. 1968. *In situ* studies on the cytochemistry and ultrastructure of a symbiotic marine dinoflagellate. *J. Mar. Biol. Assoc. UK* **48**: 349–366.
- Taylor, D. L. 1969. Identity of zooxanthellae isolated from some Pacific Tridacnidae. *J. Phycol.* **5**: 336–340.
- Taylor, D. L. 1971a. Ultrastructure of the 'zooxanthella' *Endodinium chattonii* *in situ*. *J. Mar. Biol. Assoc. UK* **51**: 227–234.
- Taylor, D. L. 1971b. On the symbiosis between *Amphidinium klebsii* (Dinophyceae) and *Amphiscolops langerhansii* (Turbellaria: Acoela). *J. Mar. Biol. Assoc. UK* **51**: 301–313.
- Trench, R. K. 1993. Microalgal-invertebrate symbioses: a review. *Endocytobiosis & Cell Res.* **9**: 135–175.
- Trench, R. K., and R. J. Blank. 1987. *Symbiodinium microadriaticum* Freudenthal, *S. goreauii*, sp. nov., *S. kawagutii*, sp. nov., and *S. pilosum*, sp. nov.: gymnodinioid dinoflagellate symbionts of marine invertebrates. *J. Phycol.* **23**: 469–481.
- Trench, R. K., and H. Winsor. 1987. Symbiosis with dinoflagellates in two pelagic flat worms *Amphiscolops* sp. and *Haplodiscus* sp. *Symbiosis* **3**: 1–22.
- Tripodi, G., and S. Santisi. 1982. A study on the cell covering of *Symbiodinium*, a symbiote of the octocoral *Eunicella*. *J. Submicrosc. Cytol.* **14**: 613–620.
- Yamasu, T. 1988. Symbiosis of marine animals and algae. *Genetics* **42**: 12–20.